

**Separation and Identification of Peptides by Supercritical Fluid Chromatography  
Coupled with Mass Spectrometry**

A Thesis  
Presented to  
The Academic Faculty

By

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## List of Symbols or Abbreviations

$^{13}\text{C}$  NMR – Carbon – 13 Nuclear Magnetic Resonance Spectroscopy

DMF – Dimethylformamide

ESI – Electrospray Ionization

GC-MS – Gas Chromatography – Mass Spectroscopy

$^1\text{H}$  NMR – Proton Nuclear Magnetic Resonance Spectroscopy

HPLC – High Performance Liquid Chromatography

ISCO – Brand Name of Syringe Pump

MeOH – Methanol

MS – Mass Spectroscopy

SFC – Supercritical Fluid Chromatography

scCO<sub>2</sub> – Supercritical Carbon Dioxide

S<sub>N</sub>2 – Bimolecular Nucleophilic Substitution

TFA – trifluoroacetic acid

TBA-Cl – tetrabutyl ammonium chloride

UV-vis – Ultraviolet-visible spectroscopy

## Abstract

The presence of certain proteins in physiological fluids could be used as an early diagnostic tool for disease; however, because of the large concentration range of proteins and the number of distinct chemical species the detection and quantification of these proteins is problematic. This research focuses on the ability to separate proteins using supercritical fluid chromatography (SFC), a form of chromatography that uses supercritical carbon dioxide (scCO<sub>2</sub>) as the mobile phase. This project was divided into two parts. The first is the synthetic aspect that involves reacting an amino acid, in this case tyrosine, with dimethyl-tert-butyl-chlorosilane which substitutes onto the hydroxyl group to increase its solubility in scCO<sub>2</sub>. The synthesis, purification, and characterization of this novel molecule have been successfully completed. The second part of the project is the optimization of the chromatograph itself, necessitating a complete rebuild of an extant SFC. Much of the internal controls were bypassed or replaced; at this stage, the SFC is capable of injecting and detecting large organic compounds and amino acids. Research efforts are now focused on separating the silylated tyrosine from nonderivatized amino acids. Once achieved, the synthesis will be scaled up to include other amino acids and ultimately small peptides, which should separate more readily and provide identification of target proteins.

# 1 Introduction

## 1.1 Background

The analysis of proteins is important in many fields of science including drug design and disease diagnosis and prevention. Approximately twenty thousand distinct protein types exist in the human body; some are present in relatively high ( $\mu\text{M}$ ) concentrations while others may be present in concentrations that are 6 or even 9 orders of magnitude less. In addition, the concentration of certain proteins, called biomarkers, may change based on the presence of disease, and accurate analysis of the presence and concentration of these biomarkers may prove to be an easy, accurate screening method for some types of disease.<sup>1</sup>

Contemporary protein analysis involves *ex vivo* digestion of the proteins and then separation and analysis of the fragments with high performance liquid chromatography (HPLC) and mass spectrometry (MS). This method suffers from several acute limitations including the length of the analysis, resolution limits, and high solvent consumption.<sup>2</sup> The solvents used in HPLC are undesirable both analytically, because of interference with MS measurements and destruction of proteins, and environmentally, because of various health and safety concerns.

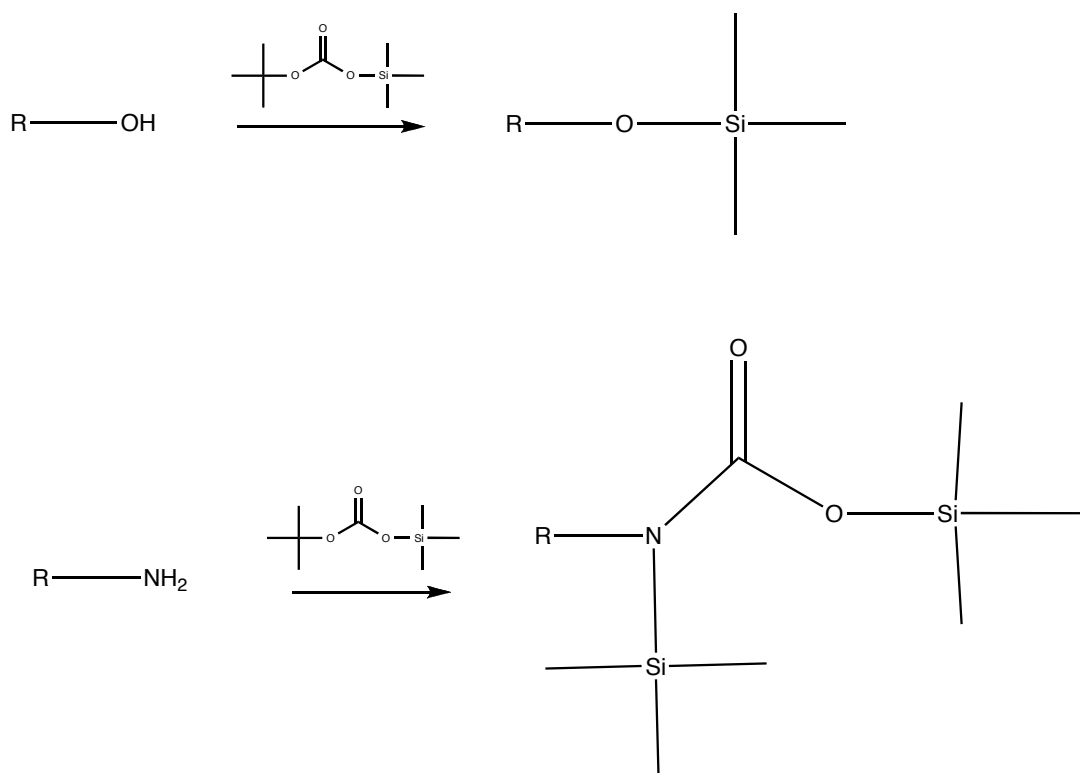
Supercritical fluid chromatography (SFC) is an analytical technique similar to both gas chromatography and high performance liquid chromatography except that instead of a gas or organic solvent, the mobile phase of SFC is a supercritical fluid – commonly supercritical carbon dioxide ( $\text{scCO}_2$ ). SFC provides several advantages over HPLC: lack

of organic solvents in the mobile phase, higher flow rates, higher temperatures, and potentially higher resolution.<sup>2</sup>

The major disadvantage of SFC is that large, relatively polar molecules, such as peptides, do not dissolve appreciably in  $\text{scCO}_2$ .<sup>2</sup> There are two strategies to increase the solubility of the peptide in the  $\text{scCO}_2$ : to increase the polarity of the mobile phase by using an additive such as  $\text{MeOH}$ <sup>2</sup> or to increase the hydrophobicity of the peptide by chemically modifying it with  $\text{CO}_2$ -philic groups.<sup>3, 4</sup> Methanol ( $\text{MeOH}$ ) was successfully used as a modifier, or co-solvent, in SFC to separate peptides of up to 40-mers long. In these experiments, trifluoroacetic acid (TFA) was used in small quantities as an additive to the  $\text{MeOH}$  to suppress the ionization of the carboxyl groups and protonate the amino groups on the amino acids. The addition of TFA increased the resolution and sharpened the peaks for all species.<sup>2</sup>

It is also possible to substitute the polar, nucleophilic functional groups on an amino acid, such as amines and alcohols, with silyl groups using an  $\text{S}_{\text{N}}2$  reaction in order to increase their  $\text{CO}_2$  solubility. The nucleophilic amino and hydroxyl groups on the amino acids will react with the *t*-butyl trimethylsilyl carbonate, as shown below.<sup>4</sup>

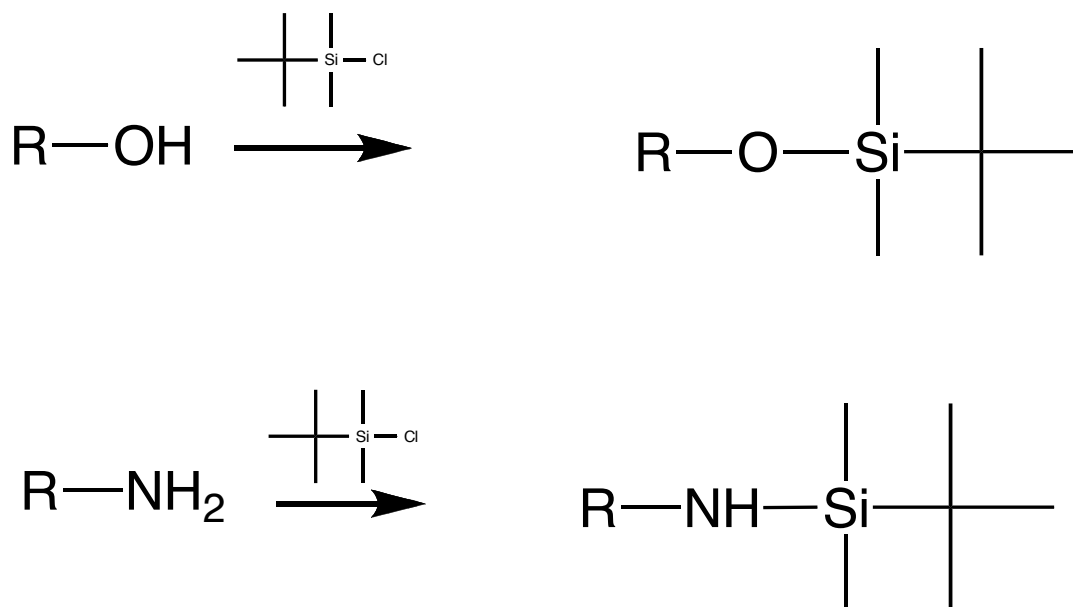




**Figure I – *t*-butyl trimethylsilyl carbonate and its adducts with nucleophilic functional groups found in amino acids**

The trimethylsilyl groups increase the hydrophobicity of the amino acids, thereby increasing their solubility in scCO<sub>2</sub>.

A similar method uses an S<sub>N</sub>2 reaction with a silyl chloride to add to the amino acids. The nucleophilic amino and hydroxyl groups attack the *t*-butyl dimethylsilyl chloride, which substitutes in much the same fashion as the *t*-butyl trimethylsilyl carbonate. The reactions are shown below.<sup>3</sup>



**Figure II -- *t*-butyl dimethylsilyl chloride and its adducts with nucleophilic groups found in amino acids**

These substitutions will have roughly the same effect on the  $\text{scCO}_2$  solubility of the amino acids as expected for the adducts of the *t*-butyl trimethylsilyl carbonate.

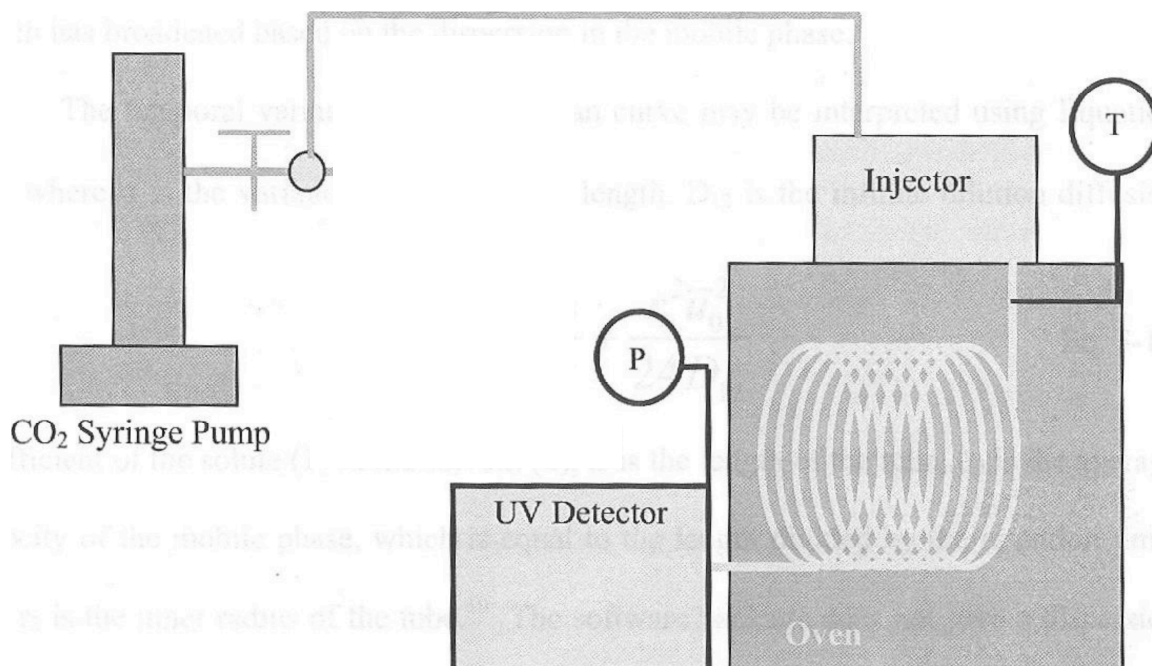
Peptides, once separated, are typically analyzed by mass spectrometry. Because of the fragile nature of peptides, electrospray ionization (ESI) is the method of choice for introducing peptides into an MS. ESI can be directly coupled to the SFC with relative ease compared to HPLC. The higher volatility of the mobile phase in SFC gives a higher efficiency of ionization of the peptide.<sup>2</sup>

## ***1.2 Introduction and Proposal of Research***

Because of the wide range of possible analytes as well as the wide concentration range over which they may be detected, any proposed analytical system must have incredible sensitivity and flexibility, be able to achieve a high rate of analysis, and be economically viable.

An SFC-MS system is an ideal way to separate, identify, and quantify peptides and small proteins. As such, this project has been an effort to rebuild and optimize an SFC extant in the laboratory as well as to chemically modify the analytes to increase their solubility in  $\text{scCO}_2$ .

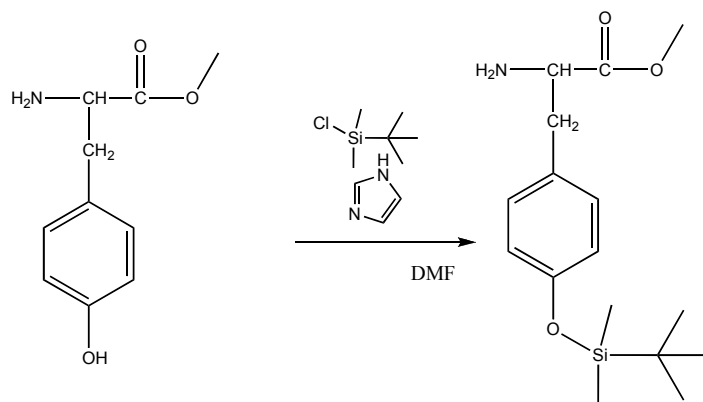
Rebuilding the SFC involves completely refurbishing the flow path, integrating the detector and injector, and replacing the entire flow control apparatus. The basic SFC design includes a syringe pump, a flow controller, a backpressure regulator, an autoinjector, a column, an oven, and a UV-vis detector. The column being used is an ethylpyridine column which has been shown to give good results separating amino acids.<sup>2</sup> The basic arrangement of components is shown in the figure below.



**Figure III -- Basic Configuration of the SFC. T is a thermocouple to monitor temperature and P is a pressure transducer.**

The modification of the analytes involves the synthesis of silylated amino acids and the determination of their solubility in scCO<sub>2</sub>. The synthesis will be performed with off-the-shelf amino acids and analyzed via gas chromatography and <sup>1</sup>H NMR. Solubility will be tested first in cyclohexane, a CO<sub>2</sub> analog in terms of polarity, via UV-vis and IR spectroscopy and later in scCO<sub>2</sub> in a high-pressure spectroscopy cell.

The proposed synthesis of the silylated tyrosine, shown below, has been demonstrated for an analogous molecule with different protecting groups.<sup>5</sup>



**Figure IV -- Synthesis of Silylated Tyrosine**

The silylated tyrosine will be a novel molecule, differing from the reported synthesis by having an unprotected amine group and a methyl-ester protecting group on the carbonyl.

Following the rebuild of the SFC and the synthesis of the silylated amino acid, the amino acids will be injected into the SFC to be separated and analyzed. If possible, these experiments will be broadened to include small peptides that will be modified and analyzed. The possible peptides being investigated are Bradykinin Fragment 1-5 and Leucine Enkephalin; their structures are shown below.



## 2 Synthesis of Silylated Amino Acids

### 2.1 Overview

Polar amino acids have very little solubility in a nonpolar solvent such as  $\text{scCO}_2$ , severely curtailing the analytical usefulness of a supercritical fluid chromatograph without a polar co-solvent. In order to increase the solubility of polar amino acids in the  $\text{scCO}_2$  mobile phase, amino acids with nucleophilic functional groups were selected to have electrophilic silyl groups added that will decrease the polarity of the overall molecule.

Currently, the supercritical fluid chromatograph possesses only an ultraviolet-visible detector, limiting the analysis to UV active molecules – the analytes must contain a phenyl group. In the first syntheses, small amino acids – lysine or cysteine – were reacted with an aromatic silyl group, and in later syntheses, an aromatic amino acid – tyrosine – was reacted with a small silyl group.

### 2.2 Synthesis Results

This section gives an overview of the results of the syntheses; for a detailed explanation of each reaction, including schemes and conditions, please see the corresponding subsection of “Experimental Methods and Materials”.

The first objective was to synthesize a silane derivative that contains a phenyl ring, which can be detected via UV-vis spectroscopy. For this, 1-[2-(4-chloromethyl-phenyl)-ethyl]-

1,1,3,3,3-pentamethyldisiloxane was successfully synthesized in 50% yield and characterized via  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, elemental analysis, and mass spectroscopy.

This molecule was then added to a light, nucleophilic amino acid; both lysine and cysteine were chosen as a starting molecule in this attempt. According to  $^1\text{H}$  NMR the reaction had proceeded, but despite various methods, the products could not be separated from the excess silyl starting material.

Because of the inability to separate the product mixtures, the silane group used above was replaced by chloromethyldiphenylsilane, a commercially available molecule. This silane was reacted with serine, and  $^1\text{H}$  NMR showed 70% conversion. It was determined by GC-MS that the product mixture contained not only product (silylated serine) and starting material (serine) but also various adducts of the chloromethyldiphenylsilane, and this mixture was unable to be sufficiently purified through extraction and distillation.

The outcomes of these experiments prompted a paradigm shift in the syntheses, and a switch was made from small amino acids and large, UV active silyl groups to large amino acids that were already UV active and small silane groups. The rationale behind this decision was that a small silane group and a large amino acid and silyl-amino acid would make the distillation easier because of the size difference between the starting material and products. In the first attempt of the new strategy, tyrosine was reacted with trimethylchlorosilane. The reaction went to completion, but the silane bond was unstable with respect to water. In response to the hydrolysis of the product, t-



butyldimethylchlorosilane was used instead of chlorotrimethylsilane for increased stability.

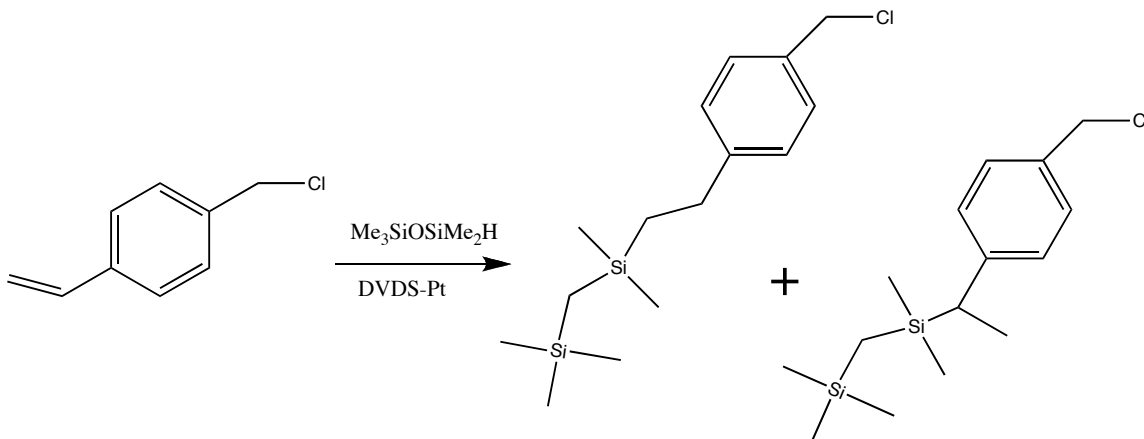
The product of the tyrosine and t-butyldimethylchlorosilane was isolated and purified in 32% yield and then characterized by  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, elemental analysis and mass spectroscopy. The characterization revealed that the tyrosine reacted with the dimethylformamide solvent, which added an aldehyde to the amine group of the amino acid. The unintended addition of the carbonyl group should not hinder, and possibly enhance, the  $\text{scCO}_2$  solubility of the molecule.

It is helpful to note that in peptides, the addition of the aldehyde to a terminal amine should occur only on the N-terminus of the peptide chain. It is hypothesized that amines in the functional group of an amino acid will be nucleophilic enough to react with the silanes, and that the amines on the N-terminus will not because they are not as electron-rich because of the proximity of the carbonyl on the  $\beta$ -carbon.

### **2.3 Experimental Methods and Materials**

All chemicals were ordered from Aldrich or VWR and used as received unless otherwise noted.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were taken using a Varian Mercury Vx-400 spectrometer with deuterated chloroform as an internal reference. Mass Spectrometry samples were submitted to the Georgia Institute of Technology Mass Spectrometry Lab for ESI-MS analysis unless otherwise noted. All elemental analyses were submitted to Atlantic Microlabs, Inc.

### 2.3.1 Synthesis of 1-[2-(4-chloromethyl-phenyl)-ethyl]-1,1,3,3,3,-pentamethyldisiloxane



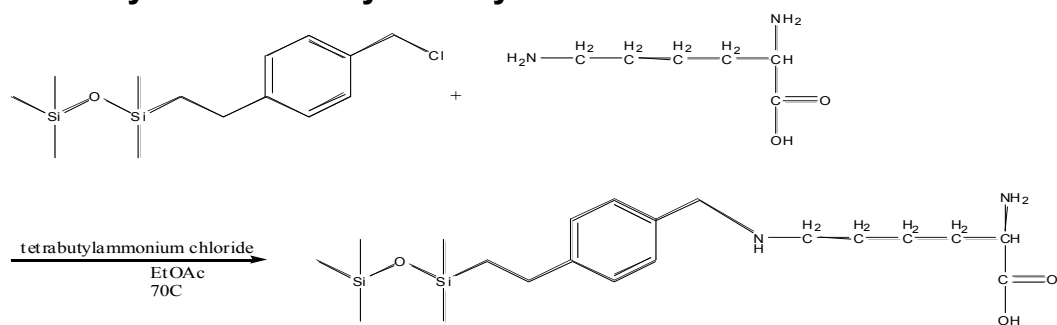
**Figure VI – Synthesis of 1-[2-(4-chloromethyl-phenyl)-ethyl]-1,1,3,3,3,-pentamethyldisiloxane. Both structural isomers are shown**

Five grams of 4-vinyl-benzyl-methyl chloride were dissolved in 20mL of heptane and placed under  $\text{N}_2$ . The solution was heated to  $75^\circ\text{C}$ , and 1.7g (1wt%) DVDS-Pt (Pt(0)-1,3-divinyl-1,1,3,3,-tetramethyldisiloxane) catalyst, 3wt% in xylene, were added. The pentamethyldisiloxane was dissolved in heptane (5.75g in 5mL) and added dropwise to the solution. The rate of addition was controlled such that the temperature of the reaction did not increase above  $77^\circ\text{C}$ . After the addition was complete, the temperature was decreased to  $70^\circ\text{C}$ , and the reaction stirred for 3 hours. The reaction was then cooled to room temperature and stirred overnight. The heptane was removed under reduced pressure, and the product was separated on a silica column with hexane as the mobile phase. The hexane was removed under reduced pressure, and the reaction gave a 50%

yield. Throughout the rest of this thesis, this compound will be referred to as Me<sub>5</sub>Si<sub>2</sub>BnCl.

1-[2-(4-chloromethyl-phenyl)-ethyl]-1,1,3,3,3-pentamethyldisiloxane: <sup>1</sup>H NMR (CDCl<sub>3</sub>) ppm: 0.1 (15, m), 0.9 (2, m), 1.3 (2, m), 2.3 (2, m), 2.7 (2, m), 4.6 (2, s), 7.2 (4, m). <sup>13</sup>C NMR (CDCl<sub>3</sub>) ppm: 1.516-2.156, 20.39, 29.2533; 46.49, 129.42-127.44, 134.50, 145.56. GC-MS analysis was done on a HP GC 6890/ HP MS 5973. MS(m/z): 300 (M<sup>+</sup>). EA: calculated, C, 55.87%, H, 8.41%. Found, C, 55.82%, H, 8.41%.

### 2.3.2 Synthesis of Silylated Lysine

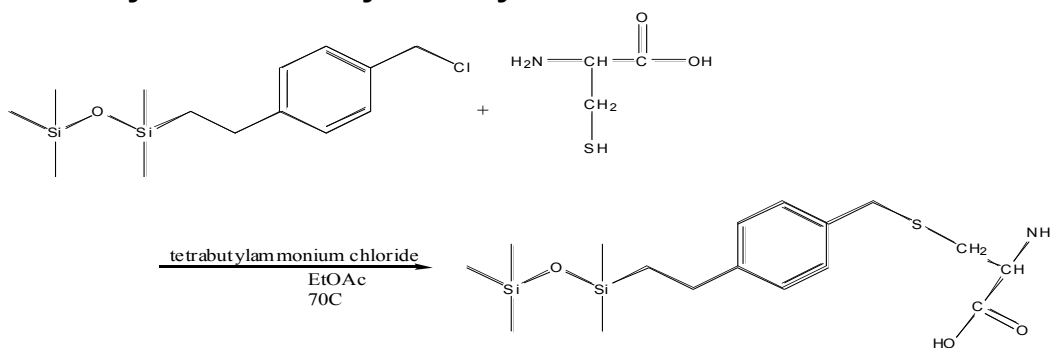


**Figure VII -- Synthesis of Silylated Lysine**

The reaction mixture consisted of 0.6mL of Me<sub>5</sub>Si<sub>2</sub>BnCl, 0.8g Lysine, 0.0247g tetrabutyl ammonium chloride (TBA-Cl) – a phase transfer catalyst – and 0.2mL of triethylamine – a proton sponge – in 6mL of ethyl acetate. The reaction was heated to 70°C under nitrogen and stirred overnight. The reaction mixture was initially separated using a liquid/liquid extraction into water. The extract was determined to be impure via GC-MS. The mixture was then separated across a silicon column using a 75:25 Hexane:Ethyl

Acetate mobile phase. An ethyl acetate/methanol flush washed both product and starting material off the column; but they could not be separated.

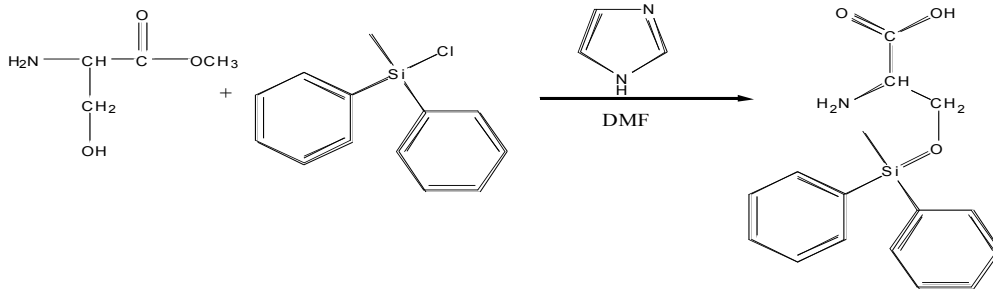
### 2.3.3 Synthesis of Silylated Cysteine



**Figure VIII -- Synthesis of Silylated Cysteine**

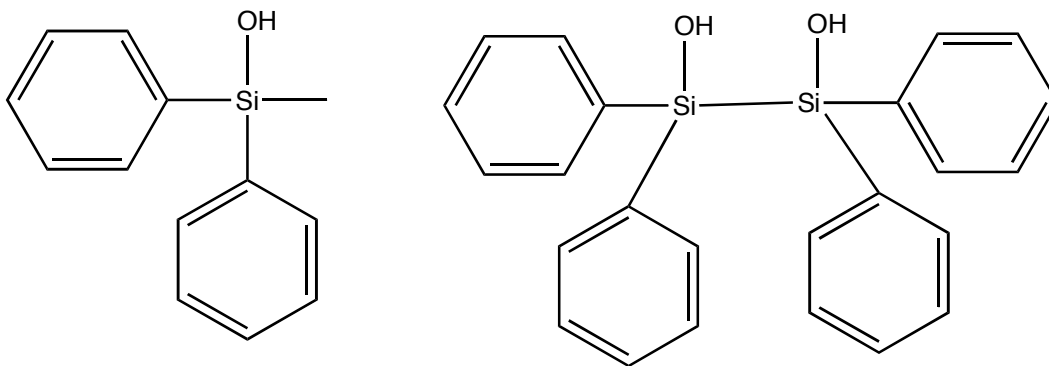
The reaction mixture consisted of 0.3mL  $\text{Me}_5\text{Si}_2\text{BnCl}$ , 0.33g Cysteine, 0.014g TBA-Cl, and 0.1mL triethylamine in 6mL ethyl acetate. The mixture was heated to 70°C and stirred for two days, after which the mixture was separated across a silica plug using a hexane/ethyl acetate mobile phase, which failed to separate the starting material from the product.

### 2.3.4 Synthesis of Silylated Serine



**Figure IX -- Synthesis of Silylated Serine**

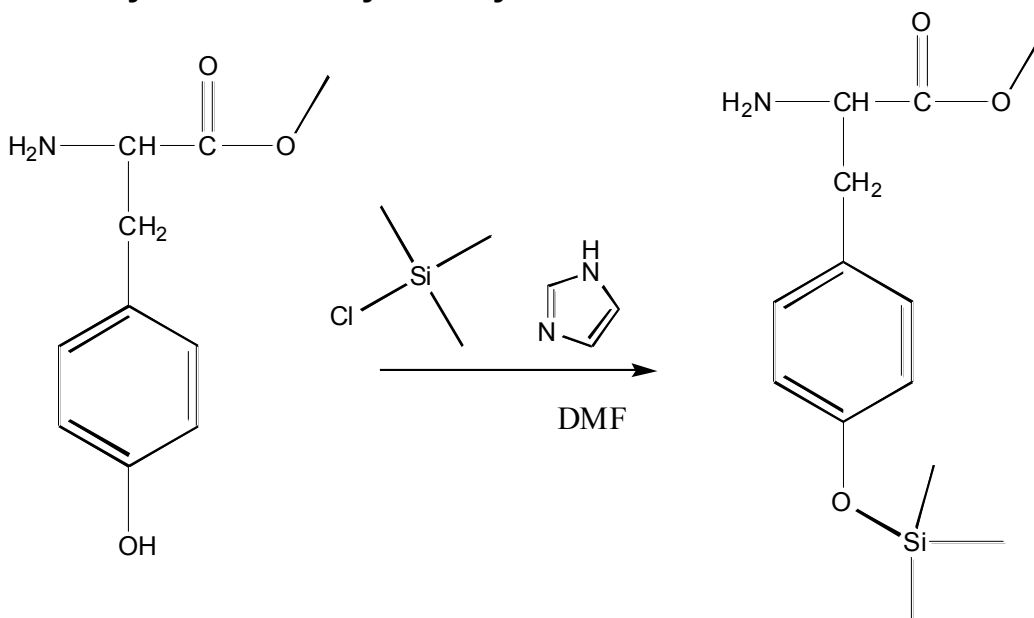
The first attempt at this synthesis had the silyl group in excess. The serine (0.5g) was combined with 0.4g imidazole and .8g chloromethyldiphenylsilane ( $\text{Ph}_2\text{MeSiCl}$ ) in 4mL dimethylformamide (DMF). The reaction was stirred under nitrogen for 4 hours at room temperature. The  $^1\text{H}$  NMR indicated that the silane had been consumed, and the mixture was dissolved in 25mL of water and extracted with 3x50mL portions of diethyl ether. The extract was analyzed by gas chromatography and found to contain product, serine, and two adducts of the  $\text{Ph}_2\text{MeSiCl}$ , shown in the figure below. This mixture could not be separated.



**Figure X -- Adducts of  $\text{Ph}_2\text{MeSiCl}$  found in the product mixture**

Because of the silyl adducts found in the product mixture, the reaction was attempted with the serine in excess to ensure the complete consumption of the silyl reagent. In this scheme, 0.4g serine, 0.3g imidazole, and 0.55g Ph<sub>2</sub>MeSiCl were combined in 6mL of DMF and stirred for 4 hours under nitrogen. The reaction mixture was dissolved in 50mL of water and extracted with 3x50mL portions of ether. The product mixture was found to contain serine by GC-MS, and the mixture could not be separated.

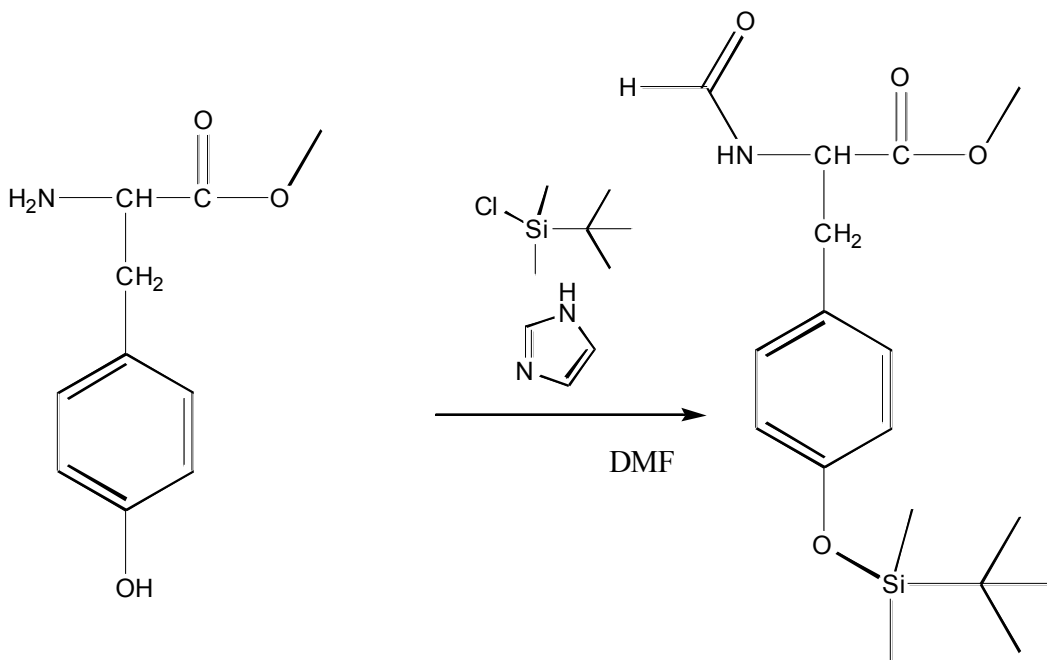
### 2.3.5 Synthesis of Silylated Tyrosine



**Figure XI -- Synthesis of Silylated Tyrosine using trimethyl silyl chloride**

The first attempt to synthesize silylated tyrosine used trimethylchlorosilane (TMS-Cl, 0.43g) with 1.4g of tyrosine and 0.5g of imidazole in 5mL of DMF. The mixture was stirred overnight under nitrogen and then dissolved in 50mL of water. It was extracted with 3x50mL portions of diethyl ether, but the product silane was not stable in water.

The synthesis was then repeated with t-butyldimethylchlorosilane (t-BuMS-Cl), which was believed to be more stable in water.



**Figure XII -- Synthesis of silylated tyrosine using t-butyldimethylchlorosilane**

For this reaction, 0.5g tyrosine, 0.7g t-butyldimethylchlorosilane, and 0.5g imidazole were combined in 10mL of DMF and stirred under nitrogen overnight. The reaction mixture was dissolved in diethyl ether and washed 5x with water and brine. The mixture was then separated over a silica plug using a 90:10 mixture of dichloromethane:ethyl acetate to remove impurities. The plug was then flushed with pure ethyl acetate to pull the product off of the silica. Silylated tyrosine was recovered as a yellow oil in 32% yield, and the IUPAC name for this compound is 3-[4-(tert-Butyl-dimethyl-silanyloxy)-phenyl]-2-formylamino-propionic acid methyl ester.

*3-[4-(tert-Butyl-dimethyl-silanyloxy)-phenyl]-2-formylamino-propionic acid methyl ester*: <sup>1</sup>H NMR (CDCl<sub>3</sub>) ppm: 0.18 (s, 6H), 0.97 (s, 9H), 3.1 (d, 2H), 3.73 (s, 3H), 4.9 (m, 1H), 6.05 (s, 1H), 6.7 (d, 2H), 6.9 (d, 2H), 8.1 (s, 1H). <sup>13</sup>C NMR (CDCl<sub>3</sub>) ppm: -4.46, 18.16, 25.62, 36.99, 51.86, 52.41, 120.19, 127.93, 130.22, 154.88, 160.38, 171.57. MS(m/z): 338.0 EA: calculated C, 60.50%, H, 8.06%, N, 4.15%. Found C, 60.37%, H, 8.22%, N, 3.99%.



## **3 Supercritical Fluid Chromatography**

### **3.1 Overview**

The use of supercritical fluid chromatography to separate peptides has been established in the literature; however, this method requires the use of approximately 30% by volume of methanol as a co-solvent to enhance the solubility of the polar peptide fragments.<sup>2</sup> This project intends to eliminate the necessity of the co-solvent for SFC analysis by synthetically modifying the peptides.

There is a SFC extant in the laboratory, but over the years it has been cannibalized and modified for use as a makeshift analytical instrument in other types of experiments, notably Taylor-Aris Dispersion. At the beginning of the project the chromatograph was in pieces, which necessitated a complete rebuild to render it functional in any capacity. The focus of this stage of the project was to rebuild the chromatograph and optimize the conditions for the separation of amino acids – the analytes under initial investigation.

### **3.2 Rebuilding the Supercritical Fluid Chromatograph**

#### **3.2.1 Initial Condition and First Steps**

The first step of rebuilding was to identify the components in the SFC and to reconstitute the flow path. The various components in the chromatograph were identified by comparison with an HPLC, labels on certain components, and visual inspection of some parts. The SFC contained a primary pump for the supercritical fluid (CO<sub>2</sub>), a secondary

pump for the co-solvent, a solvent mixer, a pressure transducer, and a backpressure regulator.

The supercritical fluid pump within the SFC was no longer in working condition because one of the glass pistons had broken into two pieces and no longer sealed the chamber.

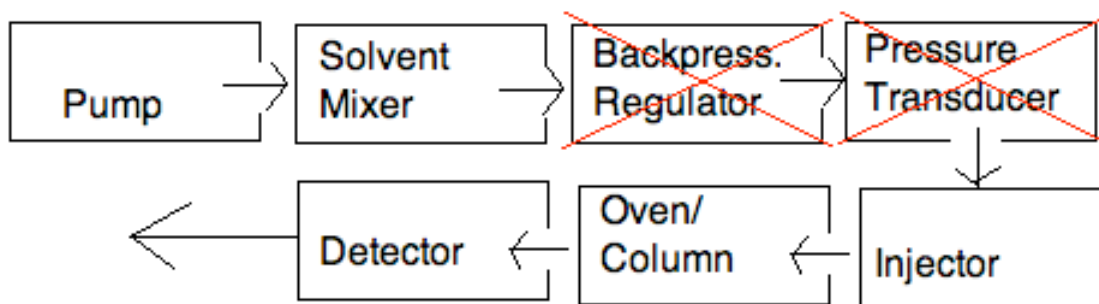
The backpressure regulator was also non-functional – the mounting bolts had been sheared off in a previous modification, and it was no longer able to hold pressure. The pump was simply replaced by an ISCO syringe pump, but the backpressure regulator was more difficult. This will be detailed in Section 3.2.2.

### **3.2.2 Injector and Detector**

The supercritical fluid chromatograph was equipped with both a computer-linked auto-injector and UV-vis detector, both of which were functional without modification. There was initially a problem with the communication between the control software on the computer and the detector; after injection, the method ceased to run and data was not recorded. The reason that the computer and the detector were unable to communicate is still not clear, but the problem was rectified by manually initiating the detector using the control panel on the face of the detector itself. This panel initiates the recording of data in real-time and starts the method run so that the temperature controls in the oven, the data recording software, and other functions of the chromatograph are controlled by the computer.

### 3.2.3 Reconstituting the Flow Path and Designing a Flow Control Apparatus

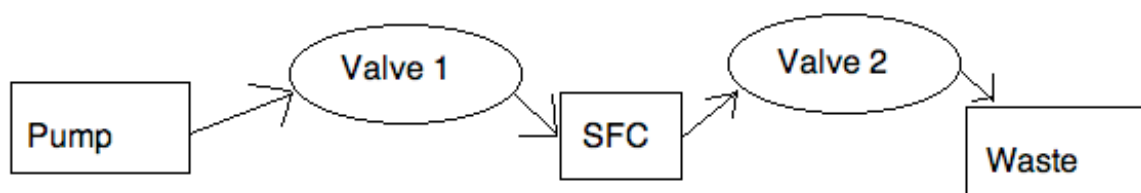
An attempt was made to repair the backpressure regulator modifying the regulator to accept new mounting bolts, and the flow path was rebuilt by running tubing from the ISCO syringe pump into the solvent mixer. The tubing then connected the backpressure regulator, the pressure transducer, and the injector in sequence. The flow path then ran from the injector through the oven and column, into the detector, and out to waste; see figure below. This flow path was blocked and would not allow CO<sub>2</sub> through; after working backwards to determine the source of the blockage, the backpressure regulator was found to be the cause.



**Figure XIII -- The initial reconstituted flow path of the SFC. The Backpressure Regulator and Pressure Transducer were removed in a later modification.**

A decision was made to completely bypass the backpressure regulator and pressure transducer in favor of devising a flow control system external to the SFC that incorporates the ISCO to control the flow rate and pressure of the CO<sub>2</sub>. This flow control system consists of two valves: the first was located immediately after the ISCO pump

before the injection assembly, and the second was after the detector at the outlet of the machine. The ISCO is run at constant pressure at 2500psig, and the first valve is used to choke down the flow to achieve a flow rate of between 0.5mL/min and 1.5mL/min, used previously in the literature.<sup>2</sup> The second valve is used to constrict the outlet to build pressure within the system to achieve a pressure in excess of 2000psig, which guarantees that the CO<sub>2</sub> is supercritical.



**Figure XIV -- Arrangement of Valves for Flow Control**

Gas expanding across a valve cools rapidly, necessitating a method of temperature control. Constant temperature ensured that the CO<sub>2</sub> would not contract, changing the flow rate and pressure of the mobile phase. Temperature was controlled by immersing the valves in water baths: the first valve was immersed in a 40°C water bath and the second in a room temperature bath. The higher temperature of the first bath is necessary because of the much larger initial pressure drop across this valve; it is a more endothermic process than the expansion across the second valve.

### **3.2.4 Preliminary Results**

Initial injections of small aromatic molecules such as toluene were made into the SFC in an effort to test the ability of the detector and column before amino acids were introduced. The analytes were detected by the UV-vis detector, but were also seen on

subsequent injections of pure methanol. The prolonged recurrence of these compounds implied that the analyte was strongly retained on the column during the experiments. Multiple injections of methanol were required to clean the column in preparation for another analyte injection.

Mixed injections of small aromatic molecules, such as toluene and acetophenone, were performed in an effort to observe a separation in time in the effluent. No separation was observed with these molecules, and the issue of the strong retention remained. Because the column was designed for amino acids and peptides, it was postulated that the small size of the molecules being used was one of the underlying issues, and for all subsequent trials amino acids were used exclusively.

### **3.2.5 Initial Injection and Analysis of Amino Acids**

Silylated tyrosine and neutralized tryptophan were injected into the SFC both alone and as a mixture. Peaks in the UV-vis spectrum were detected for each compound. In two separate trials, a small separation was seen between the silylated tyrosine and tryptophan amino acids that were injected. The peak sizes for both of these compounds were much smaller than expected, implying a lower solubility of the amino acids in  $\text{scCO}_2$  than expected. This implied a disparity between the actual pressure through the column and detector and the pressure that was measured by our pressure transducer at the inlet. This led to a closer inspection of the flow path of the SFC.

### 3.2.6 Further Investigation of SFC Flow Path

On further investigation, a second pressure gauge was added to the flow path at the outlet of the detector. This pressure gauge showed a pressure drop of approximately 1800psi from the inlet, where the original pressure gauge is located, to the outlet. The outlet pressure of approximately 600psig under these conditions demonstrated conclusively that the CO<sub>2</sub> at the outlet was not supercritical and cast doubts on the exact conditions of the mobile phase both in the detector itself and in the column during the separation.

An effort was made to increase the outlet pressure of the mobile phase by increasing the inlet pressure to the ISCO's maximum pressure of 3500psig and by eliminating as much tubing and as many valves as possible to reduce the pressure drop. These measures increased the outlet pressure to approximately 1000psig; however, CO<sub>2</sub> must be in excess of 1400psi to ensure sufficient density to assume that the solutes stay in solution.<sup>6</sup>

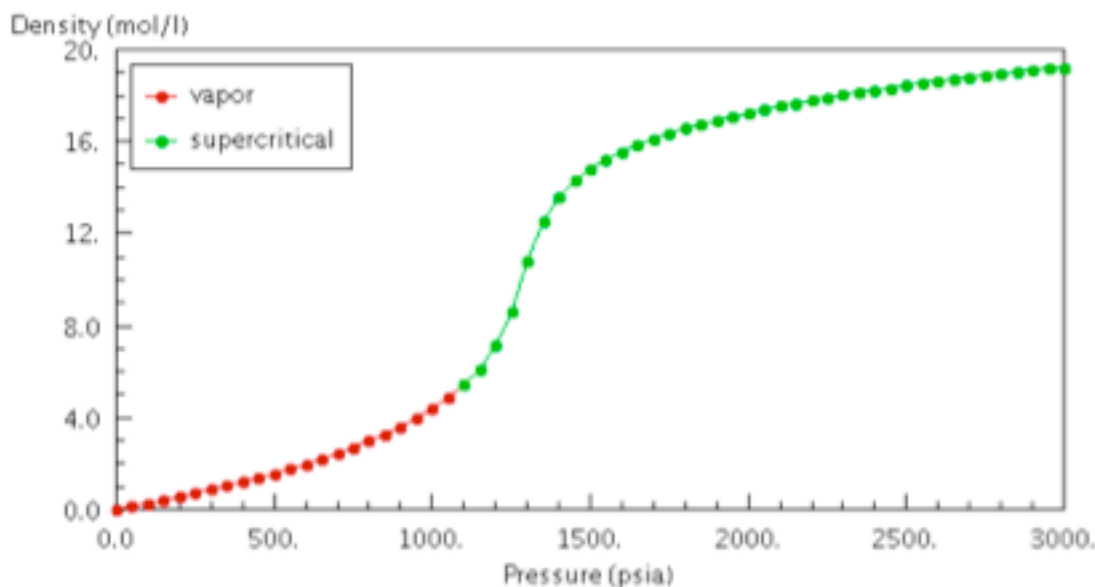
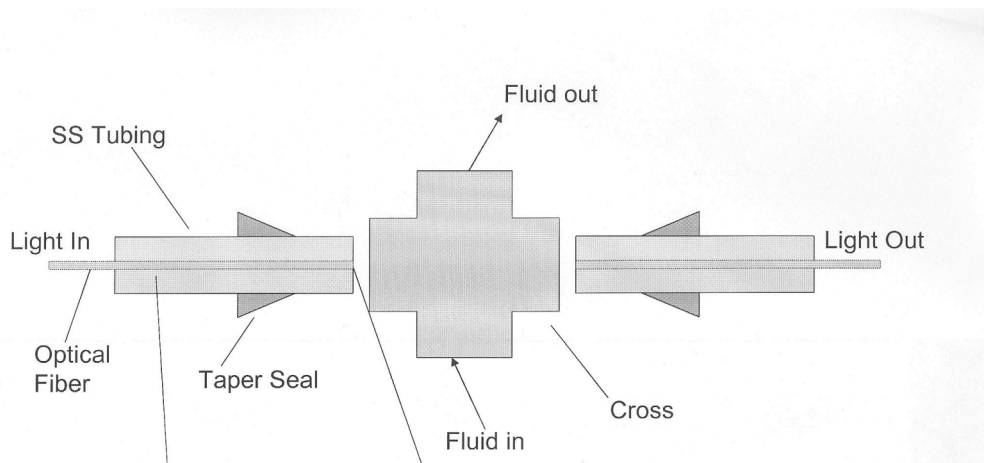


Figure XV -- Density and phase behavior of carbon dioxide at 40°C

After the outlet pressure increased to 1000psig, an existing leak in the UV-vis flow cell became both visibly and audibly present. This leak accounts for a significant amount of the pressure drop, but its location in the window of the cell required extensive repair or the fabrication of a new detector assembly.

### 3.2.7 Fabricating a New Detector

A new detector is currently being fabricated in lab using fiber-optic UV-vis light sources and detectors. The fiber-optics will be mounted in a stainless steel cross valve capable of withstanding the pressure of scCO<sub>2</sub>, and the signal will be monitored via a software program on a stand-alone computer. The fiber-optic cables will be mounted along one axis of the cross valve while the scCO<sub>2</sub> and analytes will flow across the other axis. This is diagrammed below.



**Figure XVI -- Schematic of the fiber-optic UV-vis detector apparatus**

The actual UV-vis detector, which is attached to the light out line above to detect the intensity of UV-vis radiation after it passes through the sample, is provided by Ocean Optics, as is the software used to monitor the intensity.

## 4 Conclusions

### 4.1 *Synthesis of Silylated Amino Acids*

The silylation of amino acids was successful; however, problems were encountered with the separation. It was found that separating the product of an aromatic silyl group and a comparatively light amino acid from the remaining starting materials was extremely difficult, and products were not able to be adequately purified. When a lighter silyl group was used in conjunction with a heavier amino acid, as in the synthesis of silylated tyrosine, the separation was much easier and the reaction proved useful.

When this project is scaled up to full peptides, it will not be necessary for the silyl group to have a UV signature because there will be at least one aromatic amino acid residue in the peptide. A UV signature in the starting peptide will allow the continuing use of the *t*-BuMe<sub>2</sub>SiCl reagent in the modification of peptides.

The increased mass of the peptides will also aid the separation by increasing the mass difference between the starting material and the products. If the silyl group is in excess and the peptide can be converted 100%, then a simple distillation should be able to remove the excess silylating reagent.

Because the silylating reagent is a simple electrophile, there should be no issues with scaling up the synthesis to a full peptide; each of the nucleophilic groups on the peptide



will react with the silyl group in the same manner as the individual amino acid functional groups shown above.

## **4.2 Supercritical Fluid Chromatography**

The supercritical fluid chromatograph was rebuilt to an extent that amino acids could be injected and detected. The silylation increased the solubility of the tyrosine enough to be carried through the column and detected. While a small separation of peaks was achieved, the unexpectedly small peak heights pointed to a leak in the system, which caused enough of a pressure drop so that the CO<sub>2</sub> was no longer supercritical.

Rebuilding the flow cell will allow a constant pressure flow path to be achieved under known supercritical conditions. This will ensure that the solubility of the amino acids is optimized and will allow for the determination of the effectiveness of this process. The rebuilt flow control system should, once the leak in the flow cell is eliminated, be able to adequately control the conditions of the path, and the UV-vis detector will provide a way to monitor the actual separation of the peaks. While the potential accuracy of the system has yet to be determined, it will allow for the injection and detection of amino acids and will give enough data to determine whether the separation is analytically useful.

Once the separation is optimized, the SFC platform will be connected to the AMUSE mass spectrometer interface in order to identify peptides after separation.<sup>1</sup> This will eliminate the necessity of a UV-vis detector in the process, and will remove the requirement of an aromatic residue in the peptide.

## 5 References Cited

1. Federov, A. G.; Fernandez, F. M., Focused Research Program in Nano-Engineered High Throughput Technologies for Proteomics and Biomarker Discovery. In Georgia Institute of Technology: Atlanta, 2006.
2. Zheng, J. D.; Pinkston, P. H.; Zoutendam; Taylor, *J Anal Chem* 2006, (78).
3. Mawhinney; al, e. 4,467,037, 1984.
4. Yamamoto, Y.; Tarbell, D. S.; Fehlner, J. R.; Pope, B. M., *J Org Chem* 1973, (38).
5. Hansen, D. W.; Pilipauskas, D., *J Org Chem* 1985, (50).
6. Linstrom, P. J.; Mallard, W. G., *NIST Chemistry WebBook, NIST Standard Reference Database Number 69*. National Institute of Standards and Technology, Gaithersburg MD, 20899: 2005.